APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 2002, p. 2285–2293 Vol. 68, No. 5
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.5.2285–2293.2002

Spatial Distribution of Total, Ammonia-Oxidizing, and Denitrifying Bacteria in Biological Wastewater Treatment Reactors for Bioregenerative Life Support

Yuko Sakano,[1] Karen D. Pickering,[2] Peter F. Strom,[1] and Lee J. Kerkhof[3]*

Department of Environmental Sciences[1] and Institute of Marine and Coastal Sciences,[3] Rutgers University, New Brunswick, New Jersey 08901-8521, and NASA Johnson Space Center, Houston, Texas 77058[2]

Received 30 August 2001/Accepted 11 February 2002

Bioregenerative life support systems may be necessary for long-term space missions due to the high cost of lifting supplies and equipment into orbit. In this study, we investigated two biological wastewater treatment reactors designed to recover potable water for a spacefaring crew being tested at Johnson Space Center. The experiment (Lunar-Mars Life Support Test Project—Phase III) consisted of four crew members confined in a test chamber for 91 days. In order to recycle all water during the experiment, an immobilized cell bioreactor (ICB) was employed for organic carbon removal and a trickling filter bioreactor (TFB) was utilized for ammonia removal, followed by physical-chemical treatment. In this study, the spatial distribution of various microorganisms within each bioreactor was analyzed by using biofilm samples taken from four locations in the ICB and three locations in the TFB. Three target genes were used for characterization of bacteria: the 16S rRNA gene for the total bacterial community, the ammonia monooxygenase (*amoA*) gene for ammonia-oxidizing bacteria, and the nitrous oxide reductase (*nosZ*) gene for denitrifying bacteria. A combination of terminal restriction fragment length polymorphism (T-RFLP), sequence, and phylogenetic analyses indicated that the microbial community composition in the ICB and the TFB consisted mainly of *Proteobacteria*, low-G+C gram-positive bacteria, and a *Cytophaga-Flexibacter-Bacteroides* group. Fifty-seven novel 16S rRNA genes, 8 novel *amoA* genes, and 12 new *nosZ* genes were identified in this study. Temporal shifts in the species composition of total bacteria in both the ICB and the TFB and ammonia-oxidizing and denitrifying bacteria in the TFB were also detected when the biofilms were compared with the inocula after 91 days. This result suggests that specific microbial populations were either brought in by the crew or enriched in the reactors during the course of operation.

A mission of the Advanced Life Support (ALS) Project is to develop bioregenerative life support systems to enhance the International Space Station and ultimately sustain a lunar base or a voyage to Mars. This necessitates creating a combination of physicochemical and biological systems that can regenerate resources (or materials) needed to sustain life for long time periods, possibly without the potential of resupply. Since bacteria perform recycling of various elements on Earth, it is reasonable to assume that a bioregenerative ALS system for a space vehicle or colony may also rely on bacterial activity. Understanding the microbial ecology in a biological treatment system is important for designing and operating it at optimal efficiency and to ensure reliability. In 1997, two fixed-film biological wastewater treatment reactors were tested by the National Aeronautics and Space Administration (NASA) at the Johnson Space Center (JSC) during a 91-day simulated long-term manned space mission. This experiment (Lunar-Mars Life Support Test Project—Phase III) consisted of four crew members confined in a test chamber. The system utilized an immobilized cell bioreactor (ICB) for organic carbon removal and a trickling filter bioreactor (TFB) for ammonia removal, supplemented by additional physicochemical devices for reclaiming potable water.

* Corresponding author. Mailing address: Institute of Marine and Coastal Sciences, Cook Campus, Rutgers University, 71 Dudley Rd., New Brunswick, NJ 08901-8521. Phone: (732) 932-6555, ext. 335. Fax: (732) 932-6520. E-mail: kerkhof@imcs.rutgers.edu.

In this report, we characterize the microbial community structures in the ICB and the TFB. Previously, many studies have demonstrated the existence of diverse groups of microorganisms in activated sludge and in fixed-film treatment systems (7, 8, 14, 20, 22, 23, 28, 31). However, the Phase III test at JSC represents a unique opportunity in which the microbial populations responsible for treating the wastewater (and a human crew) were allowed to develop in isolation for a 3-month period. The major questions addressed in this research include (i) which microorganisms were present in the JSC bioreactors and whether they differ from those previously reported and (ii) which microorganisms, if any, were brought in by the crew or enriched during the course of the Phase III test.

To address these questions, we characterized the total, ammonia-oxidizing, and denitrifying bacterial communities in the system by using three target genes, i.e., the 16S rRNA, ammonia monooxygenase (*amoA*), and nitrous oxide reductase (*nosZ*) genes, respectively. The *amoA* gene was chosen to identify bacteria responsible for ammonia removal (24, 25), and the *nosZ* gene (26, 27) was used to detect bacteria responsible for loss of fixed nitrogen from the wastewater treatment systems. These two functional groups (nitrifiers and denitrifiers) are essential for waste processing and resource recovery of nitrogen. A functional gene approach was selected for the N-cycling groups because of concerns regarding specificity when a 16S rRNA gene approach is used for nitrifiers

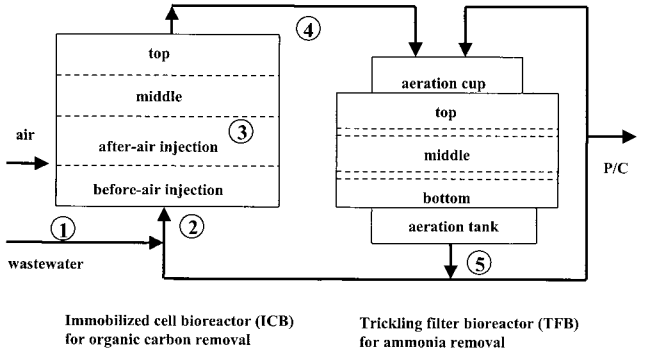

FIG. 1. Schematic of biological wastewater treatment reactors for Phase III test. Table 2 contains the wastewater characteristics at various places within the reactors indicated by the numbers. P/C, physicochemical treatment.

(24) or because the ability to denitrify is not limited to specific taxonomic groups (32). This analysis, in conjunction with physical and chemical data obtained during the Phase III test, may help improve the design and performance of biological wastewater treatment reactors for the ALS Project.

MATERIALS AND METHODS

The wastewater treatment system consisted of six subsystems, including two fixed-film bioreactors (Fig. 1), which are the target of this study, and four physicochemical reactors. The reactor structures and operating conditions are shown in Fig. 1 and Tables 1 and 2 (see reference 19 for additional information). The system was designed to process 110.6 kg of wastewater generated by the crew per day. The average total organic carbon (TOC) and ammonia removal percentages were 94 and 48%, respectively, during the manned portion of the test. The water supply was recycled 10 times during the test without any additional resupply.

ICB. An ICB was constructed by using an acrylic cylinder (25.4-cm inside diameter, 218-cm height) filled with 60 acrylic plates held 2.54 cm apart by spacers (Tables 1 and 2). Each plate was covered on both sides by a porous polymer support for microbial colonization. Wastewater entered the bottom of the reactor, and air was injected at a rate of 1.5 liters/min at a distance 16.5 cm from the bottom in an upflow cocurrent manner. This bottom layer was designed to promote denitrification, followed by aerobic carbon oxidation in the remainder of the column. The influent of the ICB was the mixture of wastewater from a feed tank and a portion of the TFB effluent (see below).

TFB. A TFB was also constructed by using an acrylic cylinder (25.4-cm inside diameter, 145-cm height) packed with alternating layers of 2.54-cm polypropylene Pall rings and 6-mm ceramic saddles to support microbial growth (Tables 1 and 2). The influent to the TFB consisted of the ICB effluent and recycled water from the TFB itself. A total of 2 liters of air per min was distributed throughout the TFB by injection at three locations: an aeration cup, the bottom of the reactor, and an aeration tank (Fig. 1). The effluent from the TFB was recycled into the TFB and ICB or processed in a series of physicochemical treatments and stored for human consumption.

Bioreactor inoculation, operation, and sampling. The ICB for organic carbon removal was inoculated with a commercial microbial consortium (DBC Plus Type ABH; Enviroflow, Inc., Manassas, Va.) on 23 August 1997 and acclimatized for 4 days. On 27 August, the ICB began receiving wastewater (shower, handwash, and urine) donated by personnel at JSC, as well as simulated humidity condensate. From 19 September to 19 December (91 days), the ICB reactor received wastewater generated by the four crew members enclosed in a sealed test chamber. The sources of wastewater were laundry, shower, handwash, oral hygiene, urine, and humidity condensate. The reactor was continuously operated throughout the phase III test at a nominal influent flow rate of 75 ml of wastewater per min and 150 ml of recycled water per min from the TFB. The average TOC concentration measured was 294 mg/liter in the feed tank, 180 mg/liter at the inlet to the ICB, and 17 mg/liter in the ICB effluent during the manned portion of the test. The concentration of organic carbon at the inlet to the ICB was lower than that in the wastewater collection tank due to the effect of dilution by the recycled water.

TABLE 1. Reactor specifications and operating conditions of the ICB and TFB

System	Vol (m^3)	Diam (m)	Ht (m)	Feed flow rate (ml/min)	Recycling flow rate (ml/min)[a]	Hydraulic retention time (min)	TOC loading rate (mg/liter · h)
ICB	0.11	0.254	2.18	75	150	1,467 (489)[b]	675
TFB	0.07	0.254	1.45	75	10,000	933 (6.9)	3.39

[a] From TFB effluent. The high rate in the TFB was used to minimize gradients and ensure aerobic conditions within the reactor.
[b] Nominal, based on empty bed volume; time for a single pass is in parentheses.

The TFB for ammonium removal was inoculated with a commercial nitrifying bacterial consortium (DBC Plus NitroTreat 20; Enviroflow) and acclimatized for 10 days. The influent was a mixture of the effluent from the ICB at 225 ml/min with the recycled flow from the aeration tank below the TFB outlet at 10 liters/min. (The high recycling rate provided a uniform distribution of wastewater throughout the reactor.) The ammonium N concentration ranged from 59 to 370 mg/liter in the influent mixture with an average concentration of 87 mg/liter in the TFB effluent.

DNA extraction and PCR amplification. Genomic DNA was extracted from biofilm samples by a phenol-chloroform procedure described in reference 25 with eight freeze-thaw cycles. Between 100 and 800 mg of biofilm or commercial inoculum was used to extract genomic DNA. For 16S rRNA gene amplification, genomic DNA (<10 ng) was amplified with eubacterial universal primers 27F and 1525R (13) as described previously (25). For ammonia monooxygenase (*amoA*) genes, amplification was done with primers A189 and A682 under the conditions described in reference 9. For the nitrous oxide reductase (*nosZ*) genes, primers Nos661F and Nos1773R were used as described previously (26, 27).

Cloning and sequence analysis. Cloning of the various target genes was done with the CLONEAMP pAMP1 system (Life Technologies, Gaithersburg, Md.) in accordance with the manufacturer's instructions. A total of 480 clones for 16S rRNA genes (four libraries from the ICB and one library from the TFB), 288 clones for *amoA* genes (two libraries from the ICB and one library from the TFB), and 288 clones for *nosZ* genes (two libraries from the ICB and one library from the TFB) were analyzed in this study. Clonal libraries were not created for the inocula. Plasmid purification of recombinant clones for sequence analysis was performed with a FlexiPrep Kit (Amersham Pharmacia Biotech, Piscatway, N.J.), and sequence data were generated with BigDye chemistry on an ABI 310 genetic analyzer (Perkin-Elmer, Foster City, Calif.). Identification of 16S sequence similarities of the small subunit (SSU) of rRNA utilized the Ribosomal Database Project (16). The BLASTN (1) and FASTA (21) programs were used for preliminary *amoA* and *nosZ* gene sequence similarity analyses, and a distance matrix with Jukes-Cantor correction was generated to examine sequence similarity among the pure cultures and uncultured microorganisms at the nucleic acid and amino acid levels. Phylogenetic tree reconstruction utilized both the maximum-likelihood (5) and neighbor-joining methods in the genetic data environment (30).

T-RFLP fingerprinting. For terminal restriction fragment length polymorphism (T-RFLP) analysis, a fluorescent dye was incorporated on the 5′ primer for each target gene (2, 4, 15, 17, 18). The fluorescently labeled product (ca. 50 ng) was digested with *Mnl*I (New England Biolabs, Beverly, Mass.) for 16S rRNA

TABLE 2. Average wastewater characteristics of the ICB and TFB[a]

Stage	TOC (mg/liter)	NH_4^+ N (mg/liter)	NO_2^- N (mg/liter)	NO_3^- N (mg/liter)	pH	Alkalinity (meq/liter)
1. Wastewater	247	150	Trace	Trace	8.44	11.7
2. ICB influent	150	153	14	11	8.58	10.0
3. ICB postaeration	93	143	5	6	8.57	10.3
4. ICB effluent/TFB influent	19	143	12	12	7.66	8.2
5. TFB effluent	12	90	35	32	6.27	1.3

[a] ICB Influent is a mixture of TFB effluent, recycled to the front of the process, and wastewater. The number for each stage (superscript) corresponds to that in Fig. 1.

TABLE 3. Number of terminal restriction fragment peaks of three target genes in the two bioreactors

Bioreactor layer	No. of terminal restriction fragment peaks		
	16S rRNA genes	*amoA* genes	*nosZ* genes
ICB inoculum	129	NA[a]	NA
Top	75	8	56
Middle	62	7	43
After air injection	110	5	50
Before air injection	111	4	57
TFB inoculum	43	3	38
Top	35	7	30
Middle	35	5	19
Bottom	28	5	34

[a] NA, not available due to the unsuccessful PCR amplification.

as described previously (6, 12) with *Tai*I (MBI Fermantas, Amherst, N.Y.) for *amoA* and with *Hin*pI (New England Biolabs) for *nosZ* fingerprints (27). (The restriction endonucleases were selected to give maximal resolution of terminally labeled fragments to known target sequences in the database.) All digestions were done in accordance with the manufacturer's instructions for 12 h. After ethanol precipitation, T-RFLP analysis was also carried out on an ABI 310 genetic analyzer with Genescan software and an internal size standard. The comparative T-RFLP data analyses were carried out by using the Combinatorial Polythetic Agglomerative Hierarchical clustering package (3; http://www.es.umb.edu/edgwebp.htm).

Nucleotide sequence accession numbers. The nucleotide sequences obtained in this study have been deposited in the GenBank database and assigned accession numbers AF390906 to AF390962 and AF390116 to AF390135.

RESULTS

T-RFLP analysis of the reactors. Initial characterization of the microbial communities at various places in the reactors relied on T-RFLP fingerprinting. Fluorescent amplifications were successful for nearly all of the samples, with the exception of *amoA* and *nosZ* for the ICB inoculum. It may be that ammonia-oxidizing and denitrifying bacteria were a small portion of the total community in this sample and were below the detection limit of the PCR assay (17, 18). An alternative explanation (PCR inhibitors) was not deemed likely given the 16S rRNA amplifications from the ICB inoculum did not demonstrate appreciable product suppression, and numerous attempts to eliminate any such inhibitors were unsuccessful.

In the T-RFLP fingerprints of the microbial communities, total peak numbers varied by a factor of 5 (range, 28 to 129) for SSU genes and by a factor of 3 for the functional genes (ranges, 3 to 8 for *amoA* and 19 to 57 for *nosZ*) (Table 3). The middle of the ICB contained the lowest number of T-RFLP peaks for the SSU and *nosZ* genes, while the anaerobic part of the ICB (before) harbored the lowest number of *amoA* peaks. In the TFB, the bottom and middle regions also enclosed the lowest numbers of SSU genes and *amoA* and *nosZ* genes, respectively. Not only were changes in absolute numbers of peaks discernible, but marked differences in profiles could also be seen between the inoculum and the various biofilm samples taken throughout the reactor at the end of the test (Fig. 2). Here, a

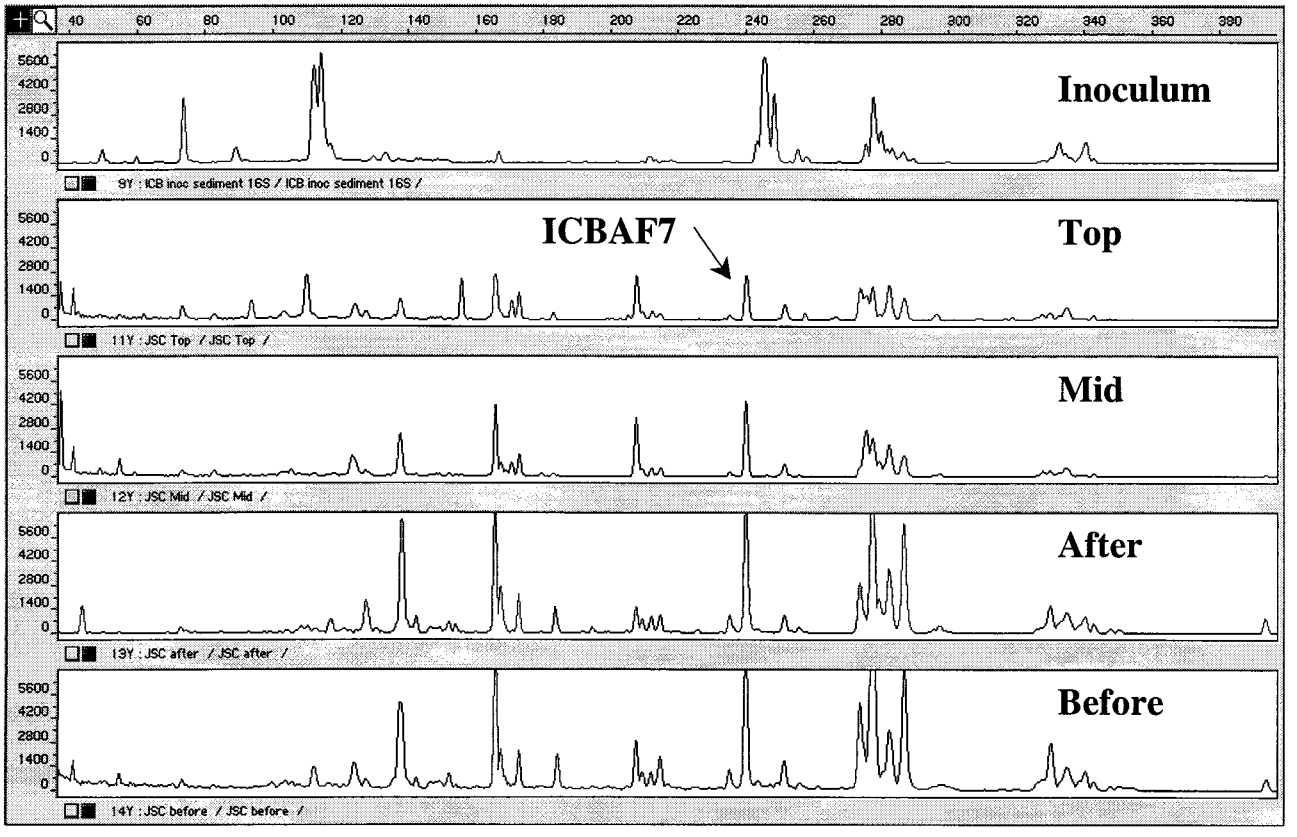
Inoculum
ICBAF7
Top
Mid
After
Before
9Y : ICB inoc sediment 16S / ICB inoc sediment 16S /
11Y : JSC Top / JSC Top /
12Y : JSC Mid / JSC Mid /
13Y : JSC after / JSC after /
14Y : JSC before / JSC before /

FIG. 2. T-RFLP profiles of 16S rRNA genes from biofilm samples of the ICB after digestion with *Mnl*I. A unique peak (clone ICBAF7) not detectable in the inoculum but found in the final samples is indicated.

TABLE 4. Comparison of the 16S rRNA genes from the clonal libraries in the ICB to the T-RFLP fingerprint of the inoculum and to sequences in the Ribosomal Database Project

Taxon and clone	Peak length (bp)[a]	Most similar organism	Sab[b]	I[c]	T[d]	M[d]	A[d]	B[d]
Proteobacteria								
Alpha group								
ICBTD9	124	*Holospora obtusa*	0.716	+	++	−+	−+	−+
ICBTE8	211	*Sphingomonas* sp. strain BRW2	0.773	+	++	−+	−+	−+
ICBMF2	288	Marine snow-associated bacterium Adriatic83	0.712	+	−+	++	−+	−+
Beta group								
ICBBA5	132	Diazotroph strain BA27	0.938	+	−−	−−	−+	+−
ICBTA10	164	*Thauera terpenica* strain 58	0.918	+	++	++	−+	++
ICBTC9	215	*Brachymonas denitrificans* strain AS-P1	0.748	+	++	−+	−+	−+
ICBBD2	214	*B. denitrificans* strain AS-P1	0.704	+	−+	−+	−+	++
ICBBB10	208	Unidentified beta proteobacterium sewCLONE2	0.836	+	−+	−+	−+	++
ICBBD7	277	Type 0803 filamentous bacterium strain Ben04B[e]	0.799	+	−+	−+	−+	++
ICBTF8	276	Unidentified bacterium strain rA1[e]	0.824	+	++	−+	−+	−+
ICBTF7	276	Uncultured bacterium clone KC208	0.784	+	++	−+	−+	−+
ICBBC5	279	Unidentified bacterium strain rA1[e]	0.789	+	−+	−+	−+	++
ICBBA10	279	Uncultured bacterium clone KC208	0.792	+	−+	−+	−+	++
ICBMD10	278	Strain SBR 2090[e]	0.864	+	−+	++	−+	−+
ICBAA12	278	Uncultured bacterium SJA-10	0.717	+	++	−+	++	−+
Gamma group								
ICBMF3	54	*Acinetobacter lwoffii* strain A391	0.837	+	++	++	−+	−+
ICBTE5	90	Iron-oxidizing lithotroph ES-1	0.664	+	++	−−	−+	−+
ICBBE7	100	Denitrifying Fe(II)-oxidizing bacterium strain BrG3	0.766	+	++	−−	−+	++
ICBTB11	126	*Serratia fonticola* strain DSM4576	0.900	+	++	++	−+	−+
Epsilon group								
ICBBA6	136	*Arcobacter cryaerophilus* ATCC 49615	0.839	+	−+	−+	−+	++
ICBAA7	139	Unidentified bacterium clone T55[a]	0.890	+	++	−+	++	++
ICBAF5	141	*A. cryaerophilus* ATCC 49615	0.908	+	−+	−+	++	−+
ICBTE4	142	*A. cryaerophilus* ATCC 49615	0.878	+	++	−+	++	−+
ICBTA1	142	*A. cryaerophilus* ATCC 49615	0.941	+	++	++	++	++
ICBTG2	289	Unidentified bacterium clone T55[e]	0.804	+	++	++	++	++
ICBTC5	288	*Arcobacter butzleri* CCUG 10373	0.742	+	++	−+	−+	−+
ICBTD7	289	*A. butzleri* CCUG 10373	0.803	+	++	−+	−+	++
ICBTC7	289	*A. butzleri* CCUG 10373	0.724	+	++	−+	−+	++
Cytophagales								
ICBTA3	44	Uncultured eubacterium WCHB1-29	0.621	+	++	−+	−+	−+
ICBAF7	241	Uncultured eubacterium WCHB1-69	0.639	−	−+	−+	++	−+
Low-G+C gram-positive bacteria								
ICBBB9	125	*Clostridium hastiforme* DSM 5675	0.731	+	−+	−+	−+	++
ICBBB7	133	*Lactosphaera pasteurii* strain KoTa2	0.856	+	−−	−−	−−	+−
ICBAI4	155	*Acetonema longum* strain AP0-1	0.638	+	−+	−+	++	−+
ICBBC4	170	*Clostridium hastiforme* DSM 5675	0.774	+	−+	−+	−+	++
ICBTA2	252	*Clostridium viride* strain T2-7	0.531	+	++	−+	−+	−+
ICBTG1	297	Bacterium ASF500	0.650	−	++	−+	−+	−+
Verrucomicrobia, ICBTG4	187	*Verrucomicrobiales* PB90-1	0.635	+	+−	+−	−−	−+
Planctomycetes, ICBMG6	384	*Isosphaera* sp. strain Schlesner 666	0.646	−	−−	+−	−−	−−
Unclassified, ICBMG3	187	Unidentified eubacterium	0.801	+	−−	+−	−−	−+

[a] Base pair length of terminal restriction fragment peak expected based on sequence.
[b] Sab value in the Ribosomal Database Project database; a value of 0.9 or greater indicates a close match to a sequence in the database.
[c] I, inoculum. No clonal library was generated. A plus or minus indicates the presence or absence of a terminal restriction fragment peak at this length.
[d] T: top; M: middle; A, after air injection; B, before air injection (layers in the ICB). Within each pair, plus or minus on the left means that sequence is present or absent in the clonal library; a plus or minus on the right indicates the presence or absence of a terminal restriction fragment peak at this length.
[e] Sequence from activated sludge reported by other researchers.

dominant T-RFLP peak is shown that corresponds to a clone (ICBAF7) not found in the inoculum (Table 4; see below). For the nitrifying population, the observed peaks in the inoculum were only of the lengths associated with *Nitrosomonas*-like *amoA* genes (177 and 282 bp), while those in the reactor represented both *Nitrosomonas*- and *Nitrosospira*-like *amoA* gene peaks (453 bp) (data not shown). Differences in the *nosZ* fingerprints between inocula and samples were observed. Some *nosZ* peaks matched the following sequences in the database: *Pseudomonas* sp.-*Sinorhizobium meliloti* (152 bp), *Achromobacter cycloclastes-Bradyrhizobium japonicum* (199 bp), *Paracoccus denitrificans* (153 bp), and *Rhodobacter sphaeroides* (203 bp).

Cluster analysis of the various T-RFLP fingerprints verified much of this visual inspection. The inocula always grouped outside the samples from the various places within the reactor (Fig 3) for all three target genes. This result suggests that many members of the community are lost during reactor operation or that most of the microbial inhabitants of the reactors are induced from undetectable levels in the inocula or introduced in the wastewater feed. Total communities based on 16S T-RFLP patterns demonstrated similarities ranging from 35 to 75% (Fig. 3) for the ICB and from 28 to 67% for the TFB, with much of the difference in similarity based on very small peaks (data not shown). The clustered profiles of the 16S rRNA genes demonstrated a separation between the bacterial communities in the ICB and the TFB, implying that different microbial populations had developed despite the recycling of effluent between the reactors.

The distributions of the ammonia-oxidizing bacteria based on the *amoA* profiles also showed a clustering pattern similar to that of the 16S rRNA genes. For the ICB, the after- and before-air injection layers clustered together (89%) and the top and middle layers clustered together (80%), with the three TFB samples also clustering (83%) (Fig. 3). In the before- and after-air injection zones of the ICB and all three zones of the TFB, the peaks of both *Nitrosomonas*-like and *Nitrosospira*-like *amoA* genes were identified; however, in the top and middle parts of the ICB, only *Nitrosomonas*-like *amoA* genes were found (data not shown). The similarity values of *nosZ* were lower than those of the other two target genes, ranging from 47 to 58% for the ICB and from 27 to 34% for the TFB. In addition, the T-RF peak profiles clustered differently from the other two genes. In the ICB, the middle-layer *nosZ* populations grouped with the before and after samples rather than with the top layer sample. In the TFB, the top and bottom samples grouped together and the middle sample was more distant.

Clonal analysis of 16S rRNA genes. To gain a better understanding of the microbial populations in the reactors, clonal libraries of 16S rRNA genes were constructed from all four layers of the ICB and from the middle layer of the TFB as described previously (12, 25). Due to the similarities of the T-RFLP profiles, only two samples from the ICB and the middle sample from the TFB were analyzed for *amoA* or *nosZ* genes. Of the 384 16S rRNA clones obtained from the ICB, 39 unique partial 16S rRNA gene sequences (541 bp) were identified. Comparison of these ICB SSU sequences to the Ribosomal Database Project database indicated low homology, with similarity (Sab) values ranging from 0.621 to 0.941 (Table 4),

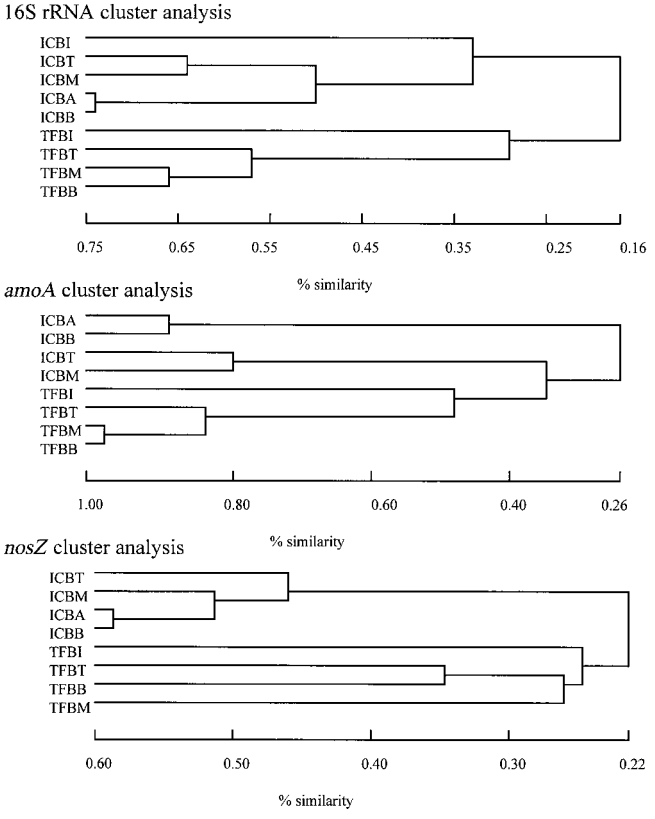

FIG. 3. Cluster analyses of the T-RFLP profiles in the ICB and TFB based on the 16S rRNA, *amoA*, and *nosZ* genes. The samples from the ICB are indicated by the following suffixes: I, inoculum; T, top layer; M, middle layer; A, after-air injection layer; B, before-air injection layer. For the TFB, the following similar suffixes are used: T, top layer; M, middle layer; B, bottom layer. The cluster analyses were performed with the Combinatorial Polythetic Agglomerative Hierarchical clustering package (COMPAH96; http://www.es.umb.edu/edgwebp.htm).

suggesting that many of the SSU genes found in the ICB have not been described in previous studies of waste treatment systems. Only five sequences showed high levels of homology (Sab values of >0.9) to sequences in the database. A total of 28 SSU clones were affiliated with the *Proteobacteria*. Low-G+C gram-positive and *Cytophagales*-like 16S rRNA gene sequences were also detected in many of the libraries. No distinct spatial distribution patterns were discerned based on the SSU genes in the different layers of the ICB.

In the TFB middle layer, analysis of 96 clones revealed 18 unique 16S rRNA gene sequences (Table 5). A total of 14 proteobacterium-like 16S rRNA gene sequences were detected (Sab values of 0.791 to 0.973), with 12 demonstrating high levels of similarity (Sab values of >0.9) to sequences in the Ribosomal Database Project. The remaining six clonal sequences were loosely related to the *Cytophaga-Flexibacter-Bacteroides* group, *Planctomycetales*, and *Nitrospira*.

Ammonia-oxidizing bacteria in the ICB and TFB based on the *amoA* genes. Among the 288 clones, a total of 27 different *amoA* genes (14 in the ICB and 13 in the TFB) were observed. Most shared high levels of sequence similarity to sequences in the database, ranging from 99.1% for *Nitrosospira* sp. strain

TABLE 5. Comparison of the 16S rRNA genes from the middle layer of the TFB to the T-RFLP fingerprints from the inoculum and other parts of the reactor and sequences in the Ribosomal Database Project

Taxon and clone	Peak length (bp)[a]	Most similar organism	Sab[b]	I[c]	T[c]	M[c]	B[c]
Proteobacteria							
Alpha group							
TFBMC2	252	*Ochrobactrum tritici* strain SCII24	0.973	+[d]	−	+	+
Beta group							
TFBMD9	208	*Dechlorisoma suila* strain PS	0.935	+	+	+	+
TFBMF4	165	Unidentified bacterium strain rJ13[e]	0.833	+	+	+	+
TFBME9	164	*Ralstonia eutropha* strain CH34	0.931	+	+	+	+
TFBMA4	219	*Alcaligenes* sp. strain NKNTAU	0.904	−	−	−	−
TFBMA5	132	Diazotroph strain BA27	0.938	−	−	−	−
Gamma group							
TFBMH1	126	*Enterobacter agglomerans* strain AH16	0.964	−	−	+	+
TFBMF5	126	*Enterobacter* sp.	0.933	−	−	+	+
TFBMB11	126	*Enterobacter agglomerans* strain AH16	0.791	−	−	+	+
TFBMH12	126	*Enterobacter agglomerans* strain AH16	0.907	−	−	+	+
TFBMH2	101	*Stenotrophomonas nitritireducens* strain L2	0.925	−	−	−	−
TFBME10	237	Unidentified gamma proteobacterium strain R2A30	0.962	−	−	−	−
TFBME4	279	*Pseudomonas* sp. strain CRE 12	0.937	−	−	−	−
TFBMC1	279	*Pseudomonas* sp. strain CRE12	0.905	−	−	−	−
Cytophagales, TFBMF3	97	Unidentified bacterium Riz1026	0.708	−	−	−	−
Nitrospira, TFBMG11	50	Unidentified eubacterium	0.802	+	+	+	+
Planctomycetes							
TFBME6	111	*Gemmata obscuriglobus* UQM446	0.554	−	+	−	−
TFBMI5	386	*Isosphaera* sp. strain Schlesner 666	0.659	−	−	−	−

[a] Length of terminal restriction fragment peak.
[b] Sab value in the Ribosomal Database Project; a value of 0.9 or greater indicates a close match to a sequence in the database.
[c] I, inoculum; T, top; M, middle; A, after air injection; B, before air injection (layers in the ICB).
[d] A plus indicates that a terminal restriction fragment peak was found at this length; a minus indicates that none found.
[e] Sequence from activated sludge reported by other researchers.

Np39-19 to 100% for *N. europaea* and were considered proof that these nitrifying microorganisms inhabited the bioreactors. However, four novel *amoA* genes were obtained that were more distantly related to *amoA* genes in the database. Two sequences (clones ICBTG2 and ICBBA2) appeared to be affiliated with the *N. europaea* cluster as defined in references 22 and 29 (Fig. 4), showing 93.5 and 93.9% similarity at the nucleotide level and 98.8 and 98.2% similarity at the amino acid level to *N. europaea*, respectively. Two other novel sequences (clones ICBBC11 and ICBBG2) grouped with the *Nitrosospira multiformis* lineage, with 92.7 and 97.1% similarity at the nucleotide level and 97.1% similarity for both at the amino acid level to *N. multiformis*, respectively. No clones were affiliated with the *Nitrosomonas eutropha* or other *Nitrosospira* branches (e.g., *N. briensis* and *N. tenuis*).

In the TFB, a total of four unique *amoA* sequences (TFBMH12, TFBME3, TFBMH4, and TFBMB10) were identified (Fig. 4). The clone TFBMH12 sequence had 94.5 and 99.4% identity with the *N. europaea amoA* gene at the nucleotide and amino acid levels, respectively. The other three sequences were close to the *N. multiformis amoA* gene, with similarities ranging from 89.9 to 92.1% and from 96.4 to 97.7% at the nucleotide and amino acid levels, respectively. The clone TFBMB10 sequence, however, appeared to be more closely related to the clone ABIAC5 sequence (97.3% identity at the nucleotide level and 98.8% identity at the amino acid level), which was present in an ammonia biofilter reported previously (25). Many of the *amoA* genes found in these bioreactors clustered at the periphery of the known groups of nitrifying bacteria.

Denitrifying bacteria in the ICB and TFB based on the *nosZ* genes. In the ICB, nine novel partial *nosZ* genes (489 bp) were obtained from the clonal libraries generated from the top and before-air injection layers, and their affiliation is shown in a reconstructed phylogenetic tree in Fig. 5. Various degrees of similarity were observed between the clonal *nosZ* gene sequences from the bioreactors and known cultured denitrifying bacteria (*Pseudomonas* sp., *Achromobacter* sp., and *Sinorhizobium* sp.) and environmental *nosZ* sequences in the GenBank database. The similarities ranged from 76.7 to 94.2% at the nucleotide level and from 68.0 to 94.2% at the amino acid level. One *nosZ* sequence, ICBBE12, was nearly identical to an environmental clone from marine sediments (696O; 99.7% identity at the nucleotide level and 100% identity at the amino acid level) (26). In the TFB, three unique *nosZ* genes were identified in the middle-layer clonal library (Fig. 5) and the clone TFBB2 sequence appeared to be related to the *P. stutzeri nosZ* gene, with similarities of 89.6% (nucleotide level) and 88.9% (amino acid level). Clone TFBD2 showed sequence similarities to the *P. fluorescens nosZ* gene of 86.5% (nucleo-

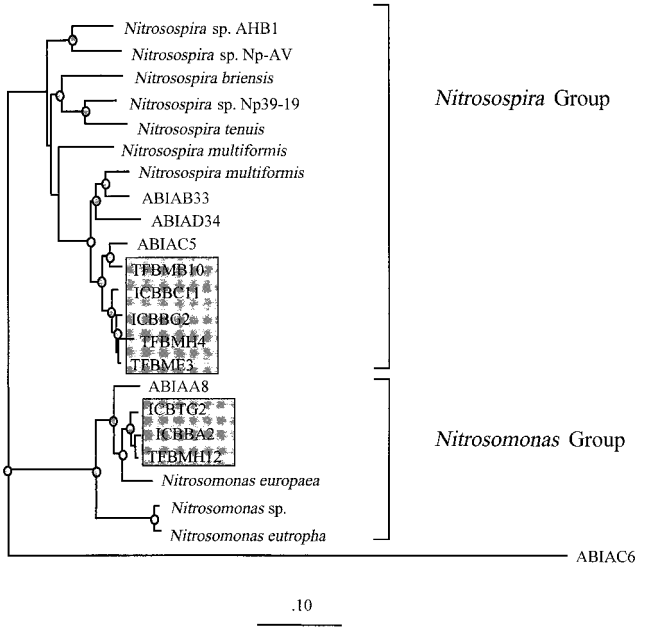

FIG. 4. Phylogenetic tree based on the partial *amoA* genes (513-bp alignment) in the ICB and TFB reconstructed by the neighbor-joining method. Nodes with more than 50 bootstrap iterations (of 100) are highlighted by circles. Clonal sequences obtained in this study are highlighted. The accession numbers of the *amoA* genes are as follows: *Nitrosomonas europaea*, L08050; *Nitrosomonas eutropha*, U51630; *Nitrosomonas* sp., AB031869; *Nitrosospira briensis*, U76553; *Nitrosospira multiformis*, AF042171; *Nitrosospira multiformis*, U89833; *Nitrosospira* sp. strain AHB1, X90821; *Nitrosospira* sp. strain Np39-19, AF006692; *Nitrosospira* sp. strain NpAV, AF016003; *Nitrosospira tenuis*, U76552; *Nitrosococcus oceani*, U96611; bioreactor samples with an ABIA prefix, AF070983 to AF070987.

tide level) and 88.9% (amino acid level). Clone TFBC2 was affiliated with the *P. denitrificans* clade at 94.7% (nucleotide level) and 94.2% (amino acid level) similarity.

DISCUSSION

The Phase III test at JSC demonstrated an integrated biologically-physicochemically based system capable of treating and recycling wastewater for consumption by a human crew (19). This analysis was carried out to obtain baseline information on the microorganisms in the reactors associated with organic carbon degradation, nitrification (ammonia oxidation), and denitrification being developed at JSC and to test whether these microbial populations are similar to those of other wastewater treatment facilities. In this study, three eubacterial divisions were found in both bioreactors: *Proteobacteria* (α, β, and γ groups), *Cytophagales*, and *Planctomycetales*. The taxonomic divisions observed in this study concur with the findings of other studies associated with wastewater (reviewed in reference 11) in finding *Proteobacteria* (α, β, and γ groups), *Cytophagales*, *Planctomycetales*, *Verrucomicrobium*, and low-G+C gram-positive bacteria. However, sequences affiliated with high-G+C gram-positive bacteria and the δ group of *Proteobacteria* reported in other wastewater studies were not detected in the JSC bioreactors. Conversely, sequences belonging to the ε group of *Proteobacteria* were found in the ICB in our study but not in the previous studies.

With respect to the spatial distribution of ammonia-oxidizing bacterial populations, the ICB top and middle layers contained only *Nitrosomonas*-like *amoA* genes and the before- and after-air injection sections harbored both *Nitrosomonas*- and *Nitrosospira*-like *amoA* genes, consistent with other reports for a bioreactor (25) and the environment (10, 24, 22, 29). The detection of *Nitrosospira*-like *amoA* may not be a surprise, in contrast to its "sporadic" detection in other wastewater treatment systems, as noted in reference 22, since biofilms may provide a greater diversity of niches compared with activated sludge. However, questions have been raised about whether a T-RFLP approach can adequately resolve *amoA* sequences, especially when using the Holmes priming set as we have in this study (22).

For this fingerprinting exercise, we used the enzyme *Tai*I, in contrast to previous work using *Taq*I, to distinguish *amoA* genes (10). Although no restriction enzyme is able to resolve all of the possible target genes for T-RFLP analysis, the *Tai*I enzyme can give peaks to diagnostic groups such as the *Nitrosomonas*-like (177 and 282 bp) and *Nitrosospira*-like (483 bp) groups. Improved resolution with T-RFLP approaches will require multiple digestions to fully resolve the various components of the different clusters. As for the Holmes priming set, in reference 22 it is reported that there exist up to six mismatches for members of the *N. europaea*-like cluster, which implies that no PCR product will be obtained when this priming set is used. However, in this and a previous study of biofilters (25), *N. europaea*-like genes have been amplified and characterized. Although these primers contain mismatches,

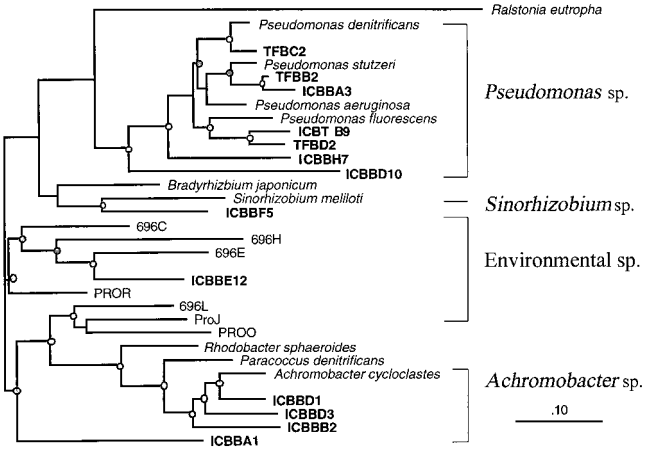

FIG. 5. Phylogenetic tree based on the partial *nosZ* genes (489-bp alignment) in the ICB and TFB reconstructed by the neighbor-joining method. Nodes with more than 50 bootstrap iterations (of 100) are highlighted by circles. Designations of clonal sequences obtained in this study start with ICB or TFB. The accession numbers of the *nosZ* genes are as follows: *Achromobacter cycloclastes*, AF047429; *Bradyrhizobium japonicum*, AJ002531; *Paracoccus denitrificans*, X74792; *Pseudomonas aeruginosa*, X65277; *Pseudomonas denitrificans*, AF016059; *Pseudomonas fluorescens*, AF056319; *Pseudomonas stutzeri*, M22628; *Ralstonia eutropha*, X65278; *Rhodobacter sphaeroides*, AF125260; *Sinorhizobium meliloti*, U47133; 696C, AF119951; 696E, AF119949; 696L, AF119927; 696H, AF119947; ProJ, AF119934; ProO, AF119933; ProR, AF119937.

these do not appear to significantly inhibit the detection of *N. europaea*-like *amoA* genes from these bioreactor samples.

Additionally, the question arises of whether these *amoA* genes belong to different species of nitrifying bacteria inhabiting these bioreactors or represent divergent copies of *amoA* genes from previously described nitrifying bacteria. Purkhold et al. suggested a threshold value of 80% similarity for *amoA* genes from different species of nitrifiers based on an extensive comparison of known strains using DNA-DNA hybridization, 16S rRNA similarity, and *amoA* similarity. None of the *amoA* genes described in this study meet this threshold criterion for novel nitrifying species (similarities in these data ranged from 90 to 97%). Although we cannot unequivocally rule out the possibility that these *amoA* genes represent novel nitrifying bacteria, it is more likely that the bioreactors harbor nitrifying strains closely related to *Nitrosomonas* and *Nitrosospira* cultures that have been characterized.

As for the denitrifying populations, the spatial distribution profiles of *nosZ* genes in the TFB differed slightly from those of the 16S rRNA and *amoA* genes, with the top and bottom portions of the reactor clustering together. Based on the *nosZ* T-RFLP profiles, the ICB appeared to harbor a more diverse group of denitrifying bacteria than the TFB. As with the 16S rRNA T-RFLP profiles, this finding is expected because of the higher total organic carbon concentrations (180 mg/liter on average, ranging from 74 to 500 mg/liter) and pH values (7.37 to 8.48) in the ICB influent than in the TFB influent (TOC of 16.6 mg/liter on average, ranging from 9 to 56 mg/liter, and pHs of 6.07 to 7.37) (19). The clonal analysis of *nosZ* genes suggests that the denitrifying bacteria that inhabit the bioreactors are generally related to cultured denitrifiers (*Pseudomonas-Paracoccus-Achromobacter* group); however (as with the *amoA* genes), the *nosZ* sequences all cluster at the periphery of the various known denitrifying microorganisms. This is in contrast to an environmental study in which few of the *nosZ* genes discovered were associated with any cultured denitrifying bacteria (26). In spite of this, three sequences were identified that were not closely affiliated with any known groups of *nosZ* genes. More attempts should be made to identify those microorganisms possessing unique *nosZ* genes from bioreactors and other samples to gain a better understanding of denitrifying bacteria in these systems.

In conclusion, the bioreactors at JSC were found to harbor unique heterotrophic (ε-*Proteobacteria*, *Cytophagales*, and *Planctomycetales*) and denitrifying populations of bacteria, but the ammonia-oxidizing populations were generally found to be closely related to known strains of nitrifiers from other wastewater treatment systems (22). This information on microbial populations is important to establish and enhance the reliability of biological water recovery systems for long-duration space flight missions. The next phase of research should focus on measuring gene expression of total and specific microorganisms, such as ammonia-oxidizing and denitrifying bacteria, to provide information about active populations in a given system (e.g., a wastewater treatment reactor). Combination of these data and quantitative data (e.g., fluorescence in situ hybridization) for these organisms with physical and chemical measurements of processes within the reactors may begin to describe the workings of what has been, until now, a "black box" system and eventually improve the design and operation of these biological systems.

ACKNOWLEDGMENTS

This work was supported in part by the New Jersey-NASA Center for Research and Training (NJ-NSCORT) and by grants from the National Science Foundation (NSF 982024) and NASA (NAG 9-1283) to L.J.K.

We thank the anonymous reviewers whose comments significantly improved the manuscript.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. L. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Avaniss-Aghajani, E., K. Jones, D. Chapman, and C. Brunk.** 1994. A molecular technique for identification of bacteria using small subunit ribosomal RNA sequences. BioTechniques **17:**144–149.
3. **Berardesco, G., S. Dyhrman, E. Gallagher, and M. P. Shiaris.** 1998. Spatial and temporal variation of phenanthrene-degrading bacteria in intertidal sediments. Appl. Environ. Microbiol. **64:**2560–2565.
4. **Bruce, K. D.** 1997. Analysis of *mer* gene subclasses within bacterial communities in soils and sediments resolved by fluorescent-PCR-restriction fragment length polymorphism profiling. Appl. Environ. Microbiol. **63:**4914–4919.
5. **Felsenstein, J.** 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. J. Mol. Evol. **17:**368–376.
6. **Garland, J. L., K. L. Cook1, J. L. Adams, and L. Kerkhof.** 2001. Culturability as an indicator of succession in microbial communities. Microb. Ecol. **42:** 150–158.
7. **Hallin, S., and P.-E. Lindgren.** 1999. PCR detection of genes encoding nitrite reductase in denitrifying bacteria. Appl. Environ. Microbiol. **65:**1652–1657.
8. **Helmer, C., S. Kunst, S. Juretschko, M. C. Schmid, K. H. Schleifer, and M. Wagner.** 1999. Nitrogen loss in a nitrifying biofilm system. Water Sci. Technol. **39:**13–21.
9. **Holmes, A. J., A. Costello, M. E. Lidstrom, and J. C. Murrell.** 1995. Evidence that particulate methane monooxygenase and ammonia monooxygenase may be evolutionarily related. FEMS Microbiol. Lett. **132:**203–208.
10. **Hortz, H. P., J. H. Rotthauwe, T. Lukow, and W. Liesack.** 2000. Identification of major subgroups of ammonia-oxidizing bacteria in environmental samples by T-RFLP analysis of *amoA* PCR products. J. Microbiol. Methods **39:**197–204.
11. **Hugenholtz, P., B. M. Goebel, and N. R. Pace.** 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. **180:**4765–4774.
12. **Kerkhof, L., M. Santoro, and J. Garland.** 2000. Response of soybean rhizosphere communities to human hygiene water addition as determined by community-level physiological profiling (CLPP) and terminal restriction fragment length polymorphism (T-RFLP). FEMS Microbiol. Lett. **184:**95–101.
13. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115–175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley & Son, Ltd., Chichester, England.
14. **Lazarova, V., D. Bellahcen, J. Manem, D. A. Stahl, and B. E. Rittmann.** 1999. Influence of operating conditions on population dynamics in nitrifying biofilms. Water Sci. Technol. **39:**5–11.
15. **Liu, W. T., T. L. Marsh, H. Cheng, and L. J. Forney.** 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl. Environ. Microbiol. **63:**4516–4522.
16. **Maidak, B. L., G. J. Olsen, N. Larsen, R. Overbeek, M. J. McCaughey, and C. R. Woese.** 1995. The Ribosomal Database Project (RDP). Nucleic Acids Res. **24:**82–85.
17. **Marsh, T. L.** 1999. Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products. Curr. Opin. Microbiol. **2:**323–327.
18. **Moeseneder, M. M., J. M. Arrieta, G. Muyzer, C. Winter, and G. J. Herndl.** 1999. Optimization of terminal-restriction fragment length polymorphism analysis for complex marine bacterioplankton communities and comparison with denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. **65:** 3518–3525.
19. **National Aeronautics and Space Administration.** 2000. Lunar-Mars life support test project: phase III final report. CTSD-ADV-341. Lyndon B. Johnson Space Center, Houston, Tex.
20. **Neef, A., A. Zalglauer, H. Meier, R. Amann, H. Lemmer, and K. H. Schleifer.** 1996. Population analysis in a denitrifying sand filter: conventional and in situ identification of *Paracoccus* spp. in methanol-fed biofilms. Appl. Environ. Microbiol. **62:**4329–4339.
21. **Pearson, W., and D. J. Lipman.** 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85:**2444–2448.

22. **Purkhold, U., A. Pommerening-Roser, S. Juretschko, M. C. Schmid, H.-P. Koops, and M. Wagner.** 2000. Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and *amoA* sequence analysis: implications for molecular diversity surveys. Appl. Environ. Microbiol. **66:** 5368–5382.
23. **Rittmann, B. E., C. S. Laspidou, J. Flax, D. A. Stahl, V. Urbain, H. Hardiun, J. J. van der Waarde, B. Geurkink, M. J. Henssen, H. Brouwe, A. Klapwijk, and M. Wetterauw.** 1999. Molecular and modeling analyses of the structure and function of nitrifying activated sludge. Water Sci. Technol. **39:**51–59.
24. **Rotthauwe, J.-H., K. P. Witzel, and W. Liesack.** 1997. The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl. Environ. Microbiol. **63:**4704–4712.
25. **Sakano, Y., and L. Kerkhof.** 1998. Assessment of changes in microbial community structure during operation of an ammonia biofilter with molecular tools. Appl. Environ. Microbiol. **64:**4877–4882.
26. **Scala, D. J., and L. J. Kerkhof.** 1999. Diversity of nitrous oxide reductase (*nosZ*) genes in continental shelf sediments. Appl. Environ. Microbiol. **65:** 1681–1687.
27. **Scala, D. J., and L. J. Kerhof.** 2000. Horizontal heterogeneity of denitrifying bacterial communities in marine sediments by terminal restriction fragment length polymorphism analysis. Appl. Environ. Microbiol. **66:**1980–1986.
28. **Schramm, A., C. M. Santegoeds, H. K. Nielsen, H. Ploug, M. Wagner, M. Pribyl, J. Wanner, R. Amann, and D. de Beer.** 1999. On the occurrence of anoxic microniches, denitrification, and sulfate reduction in aerated activated sludge. Appl. Environ. Microbiol. **65:**4189–4196.
29. **Stephen, J. R., A. E. McCaig, Z. Smith, J. I. Prosser, and T. M. Embley.** 1996. Molecular diversity of soil and marine 16S rRNA gene sequences related to beta-subgroup ammonia-oxidizing bacteria. Appl. Environ. Microbiol. **62:** 4147–4154.
30. **Smith, W. W., R. Overbeek, C. R. Woese, W. Gilbert, and P. M. Gillevet.** 1994. The genetic data environment: an expandable GUI for multiple sequence analysis. Comput. Appl. Biosci. **10:**671–675.
31. **Surampalli, R. Y., O. K. Scheible, and S. K. Banerji.** 1995. Nitrification in single-stage trickling filters. Environ. Prog. **14:**164–171.
32. **Zumft, W. G.** 1997. Cell biology and the molecular basis of denitrification. Microbiol. Mol. Biol. Rev. **61:**533–616.